CHANGES IN AMINO ACID AND SUGAR CONTENT OF BROAD BEAN LEAVES FOLLOWING INFECTION WITH *Botrytis fabae*

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ABSTRACT

Amino acid and sugar contents of healthy and *Botrytis*-inoculated bean leaves were determined. Glycine, proline, glutamic acid, DL-valine and histidine were detected in the leaves of the three bean varieties. Cystine and cystiene were detected in the leaves of the varieties "Rebaia 40" and "Giza 1". Aspartic acid, tyrosine and phenylalanine were detected only in the leaf extract of "Rabaia 40" leaves. Regarding the sugar pattern of the three broad bean varieties, sucrose, glucose, and galactose were present in appreciable amounts in the leaves of both "Rebaia 40"and "Giza 3" varieties relative to that present in "Giza 1" variety. Certain qualitative as well as quantitative changes have been detected as a result of plant infection with *Botrytis fabae*.

Leaf extracts from the three bean varieties and their individual components (except sorbose), stimulated separately the spore germination of the fungus. Leaf extract of "Rebaia 40" variety has the superior stimulatory effect followed by "Giza 3" and then "Giza 1" varieties.

INTRODUCTION

Recently it has been established that the infection of host plants with certain phytopathogenic fungi induced both qualitative and quantitative changes in their nutrient composition (Wallace *et al*., 1962; McCombs and Winstead, 1964; Tu and Ford,1970, Youssef &Youssef, 1971, Naik & Powell, 1973; Borah *et al*., (1978).

McCombs and Winstead (1964) reported qualitative as well as quantitative changes in sugars, amino acids and amides induced in cucumber fruit during the first four days of infection by *pythium aphanidermatum*.

Tu and Ford (1970) reported that the individual amino acids of soybean (Glycine max) were decreased after infection with soybean mosaic virus or bean pod mottle virus.

On the other hand, higher levels of amino acids, amides and soluble sugars were detected in tea leaves after infection with red rust alga Cephaleuros parasiticus Karst, (Borah et al., 1978).

These findings stimulated our interest to identify the amino acids and sugars in the leaves of Vicia faba L. and the parasite Botrytis fabae sardina, aiming to determine whether there were any changes in these components due to infection. Furthermore, the effect of these nutrients on spore germination of the fungus was determined to elucidate the role of them, if any, in disease development.

MATERIAL AND METHODS

Three varieties of broad bean (Vicia faba L.) namely "Rebaia 40" "Giza 3" and "Giza 1" were used in the present study. Seeds of these varieties were kindly supplied by the plant Breeding Department. Ministry of Agriculture,Giza, Egypt. Botrytis fabae culture was obtained by isolation from typically diseased broad bean leaves and plant inoculation experiments were carried out as previously described (Abu Shady et al., (1988).

Extraction and analysis of amino acids and sugars from broad bean leaves:

Amino acids:

The free amino acids were extracted from plant leaves with 80% ethanol (Thompson & Morris, 1959). Samples of 25 gm fresh leaflets from each plant set were comminuted in 100 ml ethanol for three minutes in an electric blender. The slurry was shaken for 4 hours and filtered through sintered glass, and the residue washed with four 25 ml aliquots of 80% ethanol. Extracts were stored at – 20°C until processed for chromatography.

Portions of extract were concentrated under reduced pressure at 35°C and centrifuged at 4500 g for 15 minutes. The supernatant solutions were desalted with Dowex 50 W-X resin, according to the procedure of Thompson & Morris (1959) except that the eluates were dried under pressure at 35°C. The extracted and dried amino acids were recovered and stored at 4°C in 10 % isopropanol so that 1 ml of the solution should be equivalent to 2 gm of fresh plant leaves. two dimensional chromatography on Whatman No. I paper was employed with butanol-acetic acid-water (4:1:5 v/v/v). Ordinarily 40 ul of the sample were applied to the paper.

A standard amino acid mixture, prepared according to the method of Block and Weiss (1966) was used for comparison in each test. The dried chromatograms were sprayed with 0.2% ninhydrin in acetone and then heated to 45°C for 1 hour, after which full colour was allowed to develop in a darkened chamber for 18-20 hours.

Sugars:

Samples of leaves were soaked in boiling 96% ethanol to give (with the moisture in the tissues) a final strength of 80% (10 gm F.W.+ 50 ml 96% ethanol). After boiling for 5 minutes the tissues were blended in the alcohol for 5 minutes and the macerate was then filtered. The residue was transferred to a soxhlet apparatus and extracted until no further colour appeared in the alcohol dropping down. This took nearly 4 hours as no sugars were detected in a further extraction. All the ethanolic extracts were combined and neutralized with N/10 NaOH. The extracts were then reduced to 50 ml under reduced pressure and subjected to paper chromatography. The solvent pyridine-ethylacetate-water in a ratio (20:80:10 v/v/v) was used. The chromatograms were then sprayed with aniline hydrogen phethalate reagent. A known mixture of authentic sugars was spotted for comparison on the chromatograms.

Extraction and analysis of amino acids and sugars of *Botrytis fabae* mycelium:

Erlenmayer flasks(500 ml) containing 50 ml liquid medium were inoculated with *Botrytis* spore suspension. The culture medium has the following composition: glucose 40 gm; peptone, 10 gm; potassium nitrate, 0.1 gm; potassium dihydrogen phosphate, 6.8 gm, magnesium sulphate, 2.5 gm; calcium chloride 0.1 gm; and ferric chloride, 20 mg per one litre tap water. Cultures were incubated for two weeks at 21°C and the mycelium was then filtered and washed several times with warm distilled water and then subjected to extraction and analysis.

Effect of broad bean leaf extract on Botrytis spore germination:

Spore suspension of Botrytis fabae (2 X 10^7 spores/ml distilled water) was prepared from 10 day old culture; 0.2 ml of this spore suspension were added to an equal amount of Sietz filtered leaf extract in small test tubes and incubated at 21°C for 24 hours (Buxton, 1962). The germinating spores were counted using a haemocytometer. Amino acids and sugars identified in the leaf extracts of the three broad bean varieties were similarly tested for their effect on Botrytis spore germination. Different concentrations of these components (10, 50, 100 and 500 ppm) were used. The percentage of spore germination in distilled water was used as control.

RESULTS

Amino acids determinations:

Results presented in (Table 1) demonstrate the identity and relative abundance of amino acids in the extracts of 3-week-old bean leaves of the three broad bean varieties ("Rebaia 40" "Giza 3" and "Giza 1"). It indicates that the free amino acids glycine, proline, glutamic acid, DL-valine and histidine were detected in the leaves of "Rebaia 40" "Giza 3" and "Giza 1" Cystine and cystiene were in the leaves of "Rebaia 40" and "Giza 1". Aspartic acid, tyrosine and phenylalanine were detected only in the extracts of "Rebaia 40" leaves. Tryptophan was only detected in the extract of "Giza 3" leaves. Arginine and serine were detected in the extracts of "Giza 3" and "Giza 1" plants while L-leucine was detected in "Rebaia 40" and "Giza 3" extracts.

Analysis of the amino acid content of either inoculated or uninoculated leaf extracts of 6 -week-old bean leaves (Table 2) showed that certain qualitative as well as quantitative changes in the amino acid content have occurred as a result of plant infection.

Results presented in (Table 2) have showed that threonine, ornithine, methionine, phenylalanine and L-leucine were detected only in the extract of Botrytis-inoculated "Rebaia 40" plants (the most susceptible variety). On the other hand glycine, DL-valine and traces of cystiene were only detected in the uninoculated plants of the same variety. Cystine histidine, aspartic acid, glutamic acid, proline and tyrosine were present in the extracts of both inoculated and uninoculated "Rebaia 40" plants.

Table 1. Identity and relative abundance of the amino acids and sugars spotted in extracts from healthy leaves of 3-week-old broad bean varieties.

Amino acids and sugars	Broad Bean varieties		
	"Rebaia 40"	"Giza 3"	"Giza 1"
Amino acids			
Cystine	+	–	+
Cystiene	traces	–	+++
Histidine	+++	+++	traces
Arginine	–	++++	++
Aspartic acid	++++	–	–
Serine	–	++++	++
Glycine	+++	++++	+++
Glutamic acid	++++	++++	++++
Proline	++	++	+
Tryptophane	–	++	–
DL-valine	++	++	++
Phenylalanine	++	–	–
Isoleucine	–	++	++
L-leucine	++	++	–
Tyrosine	+	–	–
Sugars			
Galactose	+	+	–
Fructose	+	+	–
Xylose	–	–	+
Maltose	++	++	++

– absent.
\+ present.
++ present in appreciable amount.
+++ present in high amount.
++++ present in very high amount.

Furthermore, the infection of the plant with the fungus Botrytis caused qualitative changes in certain amino acids (i.e glutamic acid, histidine and proline to certain extent).

Concerning the pattern of amino acids in the inoculated and uninocualted "Giza 1" variety, the results presented in (Table 2) indicated that arginine, serine, glycine, tryptophane and isoleucine were detected in the uninocualted plants. On the other hand ornithine, aspartic acid, methionine, phenylalanine, L-leucine and traces of tyrosine were present in the inoculated ones. Furthermore, it is also indicated that cystiene, cystine, histidine, glutamic acid and proline were detected in both inoculated and uninoculated "Giza 1" plants.

Results concerned with the differences in the amino acid pattern of "Giza 3" (the moderately susceptible variety) are also expressed in (Table 2). It is clear from these results that arginine, serine, glycine and tryptophane were spotted in the uninocualted "Giza 3" seedlings. On the other hand aspartic acid, L-leucine and traces of tyrosine and methionine were detected in the inoculated ones. Results also indicated that ornithine, histidine, glutamic acid and proline were present in both inoculated and uninoculated "Giza 3" plants. Quantitative changes were also observed (Table 2).

These results revealed that the amino acid pattern of the three varieties showed both qualitative and quantitative changes. The most striking changes are the presence of appreciable amounts of cystine and cystiene in the uninoculated "Giza 1" plant, compared with traces of these amino acids detected in the uninoculated "Rebaia 40" and their complete absence in the uninoculated "Giza 3" seedlings. Another interesting result was the detection of relatively great amounts of glutamic acid and histidine in the extracts of both "Rebaia 40" and "Giza 3" plants compared with their very small amounts in extract of the uninoculated "Giza 1" plant.

Analysis of the amino acids of the fungus (Table 2) show that Botrytis mycelium contained the amino acids namely: cystine, lysine, arginine, threonine, glutamic acid, DL-alanine, proline, tyrosine, tryptophane, DL-valine, phenylalanine and L-leucine.

Sugar determination:

Galactose, fructose and maltose were detected in the leaves of uninoculated 3-week-old "Rebaia 40" and "Giza 3" broad bean varieties. On the other hand, in the leaves of "Giza 1" variety, only maltose and xylose were detected (Table 1).

Concerning the sugar content of the noninoculated and inoculated 6-week-old seedlings, the results indicated that sucrose, galactose, sorbose, arabinose, fructose and maltose constitute the sugar

Table 2. Identity and relative abundance of amino acids and sugars spotted in extracts from *Botrytis* mycelium and leaves of 6- week-old healthy or infected bean varieties.

Amino acids and sugars	Broad Bean Varieties "Rebaia 40" (N)	"Rebaia 40" (I)	"Giza 3" (N)	"Giza 3" (I)	"Giza 1" (N)	"Giza 1" (I)	*Botrytis* mycelium
Amino acids							
Cystine	+	+	-	-	++	+	+
cystiene	tr	-	-	-	+++	++	-
DL-Ornithine	-	++	++	++	-	++	-
Histidine	++++	++	+++	+	tr	+	-
Arginine	+	-	+++	-	+++	-	+
Aspartic acid	+++	++++	-	+++	-	+++	-
Serine	-	-	++++	-	++	-	-
Glycine	+++	-	++++	-	++	-	-
Threonine	-	++	-	-	-	-	++
Glutamic acid	+++	++++	+++	+++	tr	+++	+++
Proline	++	+++	++	+	+	+	+
Tyrosine	++	tr	-	tr	-	tr	+
Tryptophane	-	-	++	-	+	-	+
Methionine	-	+	-	tr	-	++	-
DL-valine	++	-	-	-	-	-	+
Phenylalanine	-	+	-	-	-	+	+
Isoleucine	-	-	-	-	+	-	-
L-leucine	-	+	-	+	-	+	+
Lysine	-	-	-	-	-	-	++
Sugars							
Sucrose	+++	+++	+++	++	++	++	-
Galactose	+++	+++	++	++	++	++	++
Sorbose	+	+	+	+	++	++	+
Arabinose	+++	-	++	-	-	-	-
Fructose	++	-	+	-	-	-	-
Xylose	-	-	++	-	++	++	-
Maltose	++	+	++	+	++	++	-
Glucose	-	++	-	++	-	+	-

- absent
+ present
++ present in appreciable amount
+++ present in high amount
++++ present in very high amount
tr traces

pattern of the noninoculated "Rebaia 40" and "Giza 3" bean leaves (Table 2). The sugars of "Giza 1" variety were the same except that arabinose and fructose are completely absent. Xylose was detected in "Giza 3" and "Giza 1" bean leaves but not in "Rebaia 40" variety.

The sugars of Botrytis-inoculated bean showed more or less the same pattern except that arabinose and fructose were absent after leaf inoculation of the varieties "Rebaia 40" and "Giza 3" Furthermore, results (Table 2) indicated that glucose was detected among the sugars in the inoculated leaves of the three broad bean varieties. Similarly xylose was completely absent from the sugars of Botrytis-inoculated "Giza 3" bean leaves. On the other hand, galactose and sorbose were the only 2 sugars detected in the fungal mycelium (Table 2).

Effect of broad bean leaf extract on Botrytis spore germination:

The leaf extracts of the three bean varieties showed a stimulatory effect on the percentage of Botrytis spore germination. the percentage was 77 for "Rebaia 40" followed by 48 for "Giza 3" then 35 for "Giza 1".

Effect of the individual amino and sugars of broad bean leaf extracts on Botrytis spore germination:

The data presented in (Table 3) show that all the concentrations used (10,50,100 and 250 ppm) of amino acids and sugars (with the exception of sorbose) had a stimulatory effect on fungal spore germination, but to differing extents. Amino acids that have the superior effect were methionine, tyrosine, phenylalanine and glutamic acid in a descending order. Concerning the effect of sugars, glucose was superior followed by galactose, and then xylose and sucrose.

DISCUSSION

Although changes in host constituents have frequently been associated with disease development, interpretations of changes are usually difficult. With fungus diseases, interpretations of chemical changes must take into account additive effects of mycelium present in host tissue. In the present study, certain aspects are quite clear, others can only be speculative. Evidently most of the amino

Table 3. Effect of amino acids and sugars detected in broad bean leaf extracts on Botrytis spore germination.

Conc. of amino acids and sugars (ppm).	Spore germination after 24 hours(%)			
	10	50	100	250
Amino acids				
Cystine	25	25	27	27
Cystiene	27	32	30	32
Histidine	29	32	35	35
Aspartic acid	27	29	32	30
Arginine	30	37	32	32
Serine	25	27	25	27
Tyrosine	35	45	54	45
Tryptophane	27	27	28	27
Phenylalanine	41	48	50	45
Glutamic acid	32	37	40	38
Glycine	25	25	27	27
Proline	30	35	37	37
DL-Ornithine	27	27	30	28
Threonine	25	25	24	29
Methionine	40	62	65	63
DL-Valine	27	30	35	35
Isoleucine	29	32	37	37
L-leucine	26	26	28	28
Sugars				
Sucrose	27	38	40	29
Galactose	27	42	57	55
Glucose	30	45	60	65
Fructose	25	30	32	30
Sorbose	22	19	20	19
Arabinose	25	38	38	40
Xylose	28	40	45	45
Maltose	27	30	32	30

Distilled water 25% (Control)
Data are means of 4 replicates, 10 microscopic field each.

acids are utilized by the pathogen, as only reduced amounts were present in diseased leaves, however, some amino acids (e.g., DL-ornithine, threonine, glutamic acid, proline, methionine and phenylalanine) were detected in larger amounts than those in noninoculated ones. These could arise from several sources. Contributions to the free amino acid pool may come from proteolysis of the host proteins catalyzed by normal host or fungal enzymes. Aminc acids synthesized by the fungus also would be present. Preferential utilization of particular amino acids by the fungus in protein synthesis could account for accumulation of some amino acids in diseased leaves.

Among 18 amino acids detected and identified in the leaf extract of bean seedlings, methionine and tyrosine were superior in stimulating spore germination of the fungus. The rest of the amino acids were stimulatory or have no detrimental effect. This finding was in agreement with those of Deverall and Wood (1961b), McCombs and Winstead (1964) and Deverall (1967).

Similarly, sugars are rapidly utilized by the pathogen. However, the results in the present work did not sharply indicate this criterion since no marked differences in sugar content of inoculated and noninoculated plants were detected.

Of much interest is the absence of glucose from the sugars extracted and identified from the leaf extracts of 3-week-old bean varieties. On the other hand, glucose was detected in the leaf extract of 6-week-old bean varieties. Furthermore, glucose accomplished the most stimulatory effect on spore germination of the pathogen. These findings could explain why old leaves were more susceptible to fungal infection. That sugar was similarly reported to stimulate spore germination of fungi by Kosuge & Hewitt (1964) and Barash _et al_., (1964).

Conversely, we found that sorbose exerts an inhibitory effect on spore germination of _Botrytis fabae_ to that previously reported by Deverall (1961a).

The stimulation of spore germination of the pathogen by the whole leaf extract of 6-week-old bean varieties recorded here was identical to our field observation which showed that "Rebaia 40" was the most susceptible variety followed by "Giza 3" and the least susceptible variety was "Giza 1".

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